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THE EFFECT OF SOLVENT AND EXTRACTION METHODS ON THE BACTERIAL MUTAGENICITY OF SIDESTREAM CIGARETTE SMOKE

(Soxhlet extraction; sonication; *Salmonella typhimurium*; dichloromethane; methanol; acetone; storage)

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SUMMARY

The mutagenic activity of sidestream cigarette smoke particles was estimated by testing sidestream cigarette smoke particles which had been collected under controlled burning conditions in the laboratory. Two different extraction methods (Soxhlet and ultrasonic agitation) and 3 different solvents (dichloromethane, methanol, and acetone) were compared for their efficiencies in the extraction of compounds from sidestream cigarette smoke particles which are mutagenic in the Ames test. The mutagenic activity of the sidestream smoke particles was estimated to be 15 000–20 000 revertants per cigarette in TA98 with metabolic activation and 12 000–17 000 revertants per cigarette in TA100 without metabolic activation. Only weak mutagenic activity was detected in TA98 without activation and in TA100 with activation. Under test conditions used, ultrasonic agitation produced the most consistent results and acetone extraction produced the highest levels of mutagenic activity.

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Abbreviations: ETS, environmental tobacco smoke.

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INTRODUCTION

Environmental tobacco smoke (ETS) is composed of sidestream tobacco smoke and exhaled tobacco smoke. Sidestream smoke enters the ambient air directly from the burning tobacco source. Exhaled tobacco smoke is any combination of tobacco smoke products (mainstream and sidestream) that have been inhaled and then exhaled by an individual. The percentage of each mainstream smoke component exhaled into the environment has been reported to range from as low as 1% to as high as 70% [1,2]. The ratios of the various smoke components in sidestream smoke compared to mainstream smoke have been reported to range from 1.2- to 46-fold [3]. A typical cigarette smoker inhales mainstream smoke for approximately 3 s, 8–10 times per cigarette. This is equivalent to a total of 24–30 s out of a total average burn time of 12 min for each cigarette [4]. Sidestream cigarette smoke, therefore, is produced during 96% of the total time a cigarette is burning.

It has been estimated that sidestream smoke forms approximately 85% of the total tobacco smoke in the ambient indoor air [5]. Therefore, both smokers and non-smokers could be exposed, especially in indoor spaces with low levels of air exchange, to significant levels of airborne pollutants.

Although a great deal of effort has been expended characterizing the harmful constituents of mainstream tobacco smoke, much less is known about the toxic components of sidestream tobacco smoke. Rickert et al. [6] tested 15 different brands of cigarettes and reported that in most cases considerably more tar, nicotine, and carbon monoxide were present in the sidestream than in the mainstream smoke. The findings from a 1983 study demonstrated that indoor air contaminated with sidestream cigarette smoke can be more mutagenic than ambient outdoor air in an urban area [7]. It was reported that the smoke from one cigarette which is diluted in a structure with a volume of 300 m^3 would result in mutagenic activity 2.5 times that found in urban ambient air.

More than 2000 compounds have been identified in mainstream tobacco smoke [8]. Since sidestream smoke would be expected to be similarly complex, it is difficult to identify the specific mutagenic constituents. A recent report by the National Research Council [9] says 'Research is needed to standardize both the collection and evaluation of ETS so that the effects of ETS can be studied in the laboratories'. They also recommend that 'Further in vitro assays of ETS are needed'. The purpose of this paper is to examine and compare appropriate methods for preparing sidestream smoke samples for mutagenicity studies.

MATERIALS AND METHODS

Collection of samples

Sidestream cigarette smoke particles were collected by allowing a generic brand (Price Breaker, Winn-Dixie Stores, Jacksonville, FL) of king-size filter cigarettes

(738.5 mg tobacco/cigarette, average of 10 cigarettes), to burn in a Plexiglas chamber measuring 32 cm × 38 cm × 29 cm (volume = 0.0353 m^3). The air inside the chamber was exhausted by a constant flow vacuum pump at a rate of 0.028 m^3/min (1 cu. ft. of air per min). The air was pulled through a 142 mm diameter Teflon-coated glass fiber filter (Pallflex Products Corp., Putnam, CT) supported in a stainless steel filter stand. Filtered air was drawn into the chamber through three 1 cm diameter openings in the bottom of the chamber.

Before sample collection, all filters were heated for 24 h at 175°C and then desiccated for 24 h. After sampling, each filter was folded to enclose the sample and rewrapped in aluminum foil. All samples were returned to the laboratory after collection, usually within 2 h. To minimize sample degradation or reaction, samples were stored on the filters in the dark at −70°C.

Sample preparation

During preparation of the samples for mutagenicity testing, all solvents were glass distilled, high purity, spectroscopic grade (Burdick and Jackson, Muskegon, MI). To reduce artifact contamination only glass, stainless steel, and Teflon equipment was used in the preparation of samples. All materials were washed with Alkonox and consequently rinsed with distilled water, 50% nitric acid, 50% sulfuric acid, distilled water, methanol, and extraction solvent.

Ultrasonic extraction. Each 142 mm diameter filter was divided into 8 replicate pieces using stainless steel scissors. Each replicate was placed in a 50 ml glass vial with a Teflon-lined screw cap along with 25 ml of solvent. One of 3 different solvents (dichloromethane, methanol, or acetone) was used to extract the organic material from the filter pieces. The vials were held in a plastic rack and placed in a water bath sonicator (Sonicor, Model 401, Clean Room Products, Bay Shore, NY) for 15 min at 25°C. Solvent was then decanted into a clean vial. The samples were reextracted for an additional 15 min with 25 ml of fresh solvent. After the second sonication, the extracts were combined to yield a sample volume of approximately 50 ml.

Soxhlet extraction. Each replicate was placed in a 500 ml extraction flask with 250 ml of solvent and extracted for 24 h at a rate of 2–3 cycles/h using a standard Soxhlet apparatus (Kontes Glassware, Vineland, NJ).

Refluxing of cigarette smoke extracts. Additional filters containing sidestream cigarette smoke particles were extracted with acetone using the sonication procedure above. The extracts were then split into 4 replicates. Three of the 4 replicates were placed in individual 500 ml extraction flasks with 100 ml of acetone. The flasks were attached to a Soxhlet extractor and refluxed for 8, 16, and 24 h respectively.

Sample concentration and solvent exchange. After each of the above procedures, the extraction solvent was removed by rotary evaporation in individual 250 ml round-bottom flasks. The samples were placed in a 40°C water bath during the evaporation procedure. Each sample was concentrated to a volume of approximate-

ly 5–10 ml and then quantitatively transferred into 15 ml volumetric tubes. The samples were concentrated to a volume of 1–2 ml by immersing the tubes in a 45°C water bath while a stream of dry nitrogen was bubbled through the sample using stainless steel needles. A known volume of DMSO was added to each sample and the remaining extraction solvent was evaporated using the nitrogen purge method. Representative samples were analyzed by gas chromatography prior to bioassay to check for adequate removal of the extraction solvent. In all cases, an unused filter from each lot was extracted to determine background levels of mutagenic activity due to the filter.

Salmonella mutagenicity testing

The *Salmonella typhimurium* plate incorporation assay was performed as described by Ames et al. [10], with 2 modifications consisting of: (1) adding a trace amount of histidine to the agar base layer instead of the overlay agar and (2) incubating the plates for 72 h instead of 48 h. All samples were tested using Ames *Salmonella typhimurium* tester strains TA98 and TA100 with and without exogenous metabolic activation (S9) unless otherwise indicated. The S9 was prepared in the manner described by Ames et al. [10] from Aroclor-induced, male CD-1 rats Tester strains were obtained from Dr. Bruce Ames, University of California, Berkeley, CA and maintained by the Genetic Bioassay Branch, Genetic Toxicology Division, Health Effects Research Laboratory, U.S. EPA Environmental Research Center, Research Triangle Park, NC. All dose levels were run in triplicate, unless otherwise noted. Negative controls for spontaneous reversion and positive controls for each strain were performed in triplicate for each experiment. The control chemicals used were 2-aminoanthracene for both strains with metabolic activation. Without metabolic activation, 2-nitrofluorene and sodium azide were used as positive controls for TA98 and TA100 respectively. Each sample was tested at 4–5 doses (5, 10, 50, 100, 300 μl/plate). In most cases, these doses corresponded to 0.008, 0.016, 0.08, 0.16 and 0.48 cigarettes respectively.

All plates were counted using an automatic plate counter (Artek Model 880, Artek Systems Corp., Farmingdale, NY). Each plate was counted 3 times and a mean for the 3 counts was recorded. Data analysis was accomplished using the Ames test curve fitting program of Stead et al. [11]. This program is a FORTRAN program which fits a non-linear dose-response curve to the plate count data. Using the model slope obtained from the dose-response curve, an estimate of mutagenicity was calculated and expressed as the number of revertants per cigarette.

RESULTS

Effect of extraction method and solvent

Tables I and II present the bioassay results of the experiments designed to compare the efficiencies of 2 different extraction methods and 3 different extraction

TABLE I

THE EFFECT OF EXTRACTION METHOD AND SOLVENT ON THE MUTAGENIC ACTIVITY OF SIDESTREAM CIGARETTE SMOKE PARTICLES TESTED IN *SALMONELLA TYPHIMURIUM* TA98 WITH METABOLIC ACTIVATION

Average of 4 experiments, 5 doses, 3 replicates per dose, analysis by the method of Stead et al. [11].

Extraction method	Number of revertants (thousands) per cigarette: as a function of extraction solvent		
	DCM[a]	MEOH[a]	ACET[a]
Soxhlet	12.2 ± 10.2	10.9 ± 7.5	11.4 ± 5.1
Ultrasonic	8.0 ± 3.2	11.3 ± 3.7	14.6 ± 1.8

[a]DCM, dichloromethane; MEOH, methanol; ACET, acetone.

TABLE II

THE EFFECT OF EXTRACTION METHOD AND SOLVENT ON THE MUTAGENIC ACTIVITY OF SIDESTREAM CIGARETTE SMOKE PARTICLES WHEN TESTED IN *SALMONELLA TYPHIMURIUM* TA100 WITHOUT METABOLIC ACTIVATION

Average of 4 experiments.

Extraction method	Number of revertants (thousands) per cigarette: as a function of extraction solvent		
	DCM[a]	MEOH[a]	ACET[a]
Soxhlet	0.7 ± 1.0	0.7 ± 1.2	1.9 ± 3.6
Ultrasonic	4.9 ± 3.7	0.4 ± 0.2	14.6 ± 6.5

[a]DCM, dichloromethane; MEOH; methanol; ACET, acetone.

TABLE III

THE EFFECT OF REFLUXING ON THE MUTAGENIC ACTIVITY OF SIDESTREAM CIGARETTE SMOKE PARTICULATE EXTRACT

Number of hours refluxed	Number of revertants (thousands) per cigarette: *S. typhimurium* strain/activation conditions			
	TA98/+ S9	TA98/− S9	TA100/+ S9	TA100/− S9
0[a]	19.6	0.5	3.4	14.6
8	15.3	1.9	3.8	1.9
16	17.2	3.8	4.4	3.6
24	16.2	1.1	3.6	3.6

[a]Extract tested for activity without refluxing.

TABLE IV

THE EFFECT OF STORAGE ON THE MUTAGENICITY OF SIDESTREAM CIGARETTE SMOKE PARTICLES

Storage conditions and time	Number of revertants (thousands) per cigarette	
	TA98 + S9	TA100 − S9
Collected, extracted, bioassayed in less than 6 days	17.1	14.6
Extract stored frozen at −70° C in DMSO for 23 days	15.9	3.3
Extract stored frozen at −70° C in DMSO for 60 days	5.1	1.7
Particles stored frozen at −70° C in DMSO for 60 days	9.3	2.1
Particles stored frozen at −70° C in DMSO for 180 days	11.5	0.4

solvents. For TA98 results with metabolic activation (Table I), the differences between the 2 methods and 3 solvents were small. The activity in TA100 without metabolic activation (Table II) was almost equal to that in TA98 with activation, but only when ultrasonic agitation and acetone were used to extract the particles.

Effect of refluxing cigarette smoke extracts

Table III contains the mutagenicity data from the experiments conducted to determine whether or not the refluxing of sidestream cigarette smoke extracts affects their mutagenic activity. The mutagenic activity in TA98 with metabolic activation was not significantly affected by refluxing the extracts for up to 24 h. An increase in activity in TA98 without metabolic activation was seen after refluxing. Significant reductions in activity, however, were observed in TA100 without metabolic activation after the extracts were refluxed.

Effect of storage time and conditions

Sidestream cigarette smoke particles and extracts were stored for different lengths of time as either particles or as extracts in DMSO. Following storage, the samples were tested for mutagenic activity in the Ames test. The results of this experiment are contained in Table IV.

Substantial reductions in activity were observed in both TA98 with activation and in TA100 without activation. The largest reductions in activity were seen in TA100 without metabolic activation after the extracts had been frozen for 180 days in DMSO. The reduction in activity in TA98 was much less than the reduction in TA100 especially when the samples were stored as particles rather than as extracts in DMSO.

DISCUSSION

As early as 1974, Kier et al. [12] demonstrated the mutagenicity of mainstream

cigarette smoke condensate in *Salmonella typhimurium* strain TA1538 with a metabolic activation system prepared from polychlorinated biphenyl-induced rat liver tissue. Since 1974, numerous investigators have reported mutagenic activity for mainstream cigarette smoke and cigarette smoke condensates in a variety of mutagenicity test systems [14–18]. More recently, extracts of sidestream cigarette smoke particles have also been shown to be mutagenic in the Ames test [7,20]. As in previous studies [7], the highest mutagenic response in this study (15 000–20 000, revertants per cigarette) was observed in TA98 with metabolic activation (Table III).

Method of extraction

Although Soxhlet extraction is the most widely used procedure for the removal of organic compounds from airborne particulate samples, it has potential for artifact formation and is a lengthy procedure usually requiring a minimum of 24–36 h. During this extraction process there is opportunity for sample loss due to volatilization. In addition, since this method requires a substantial amount of glassware and laboratory hood space, it is often not practical when numerous samples are extracted in a short period of time. Soxhlet extraction also has been reported to result in the decomposition of some known mutagenic compounds [21–23].

Ultrasonic agitation, an alternate method for the extraction of airborne particles, has been reported to yield recoveries which are comparable to Soxhlet extraction [23–26]. Although this method requires less time, glassware, and supplies, the most important advantage of sonication may be the reduction in the opportunity for sample loss. A number of extraction solvents have been used in both methods including benzene, methanol, acetone, dichloromethane, acetonitrile, ethanol, and mixtures of 2 or more of these solvents [27].

A number of studies have compared these extraction methods for ambient air particles [24,25,28–31]. In a recent investigation, Rives [32] compared the efficiencies of sonication and Soxhlet extraction of woodsmoke particles. Using both mutagenic activity and extractable mass as the criteria for his evaluation, he concluded that either method can be used for the extraction of mutagenic compounds from woodsmoke. Despite individual preferences for one method over another, the recent literature recommends both techniques equally [26,33].

In this study (Tables I and II), the mutagenicity data indicate that the ultrasonic extraction method is as effective or more effective than the Soxhlet procedure in extracting those compounds from sidestream cigarette smoke particles which are mutagenic in the Ames test. The use of sonication also resulted in more consistent data than that obtained when Soxhlet extraction was used. These findings are in agreement with Sawicki et al. [28] who reported that ultrasonic agitation was a more reproducible extraction method than Soxhlet extraction.

We conclude that both Soxhlet extraction and ultrasonic agitation are effective in the extraction of mutagens from sidestream cigarette smoke particles. Sonication

requires less time and equipment and results in less opportunity for loss of sample during extraction. Thus, sonication would be the favored method in cases where large numbers of samples require extraction in a short period of time.

The choice of a solvent for use in any extraction procedure is usually influenced by the class or classes of compounds for which extraction is desired. Numerous solvents have been used by different investigators for the extraction of organic compounds from air particulate samples for mutagenicity testing in the Ames test [27,34]. Acetone has been widely used by several investigators for air particle extraction; however, some do not recommend its use because of its reactivity with other chemicals and its unavailability in pure form [35]. Other solvents and combinations of solvents, such as cyclohexane [36], benzene/dichloromethane mixture [37], cyclohexane, dichloromethane and acetone serially [38], methanol [39], and benzene [40] have also been used for the extraction of mutagenic material from air particles. Jungers and Lewtas [30] concluded that the preferred solvent for those studies in which the non-mutagenic and sometimes bactericidal mass is to be minimized is dichloromethane. Krishna et al. [25] compared 7 solvents or combinations of solvents and reported that sequential extraction with acetone followed by dichloromethane gave a higher response than acetone alone or acetone in combination with dichloromethane. Of the solvents tested by Krishna et al. [25], cyclohexane resulted in the lowest mutagenic response. Studies published by Jungers et al. [29] and Talcott and Wei [41] report similar findings. In a recently published study, acetone was the extraction solvent chosen by Alfheim and Ramdahl [20] in their study which investigated the mutagenicity of wood and sidestream cigarette smoke. In a comparison of 4 solvent systems, Talcott and Wei [41] using both Soxhlet and ultrasonic reported that acetone extracted more mutagens than benzene, chloroform, and methanol. Using both Soxhlet and ultrasonic extraction methods, Goto et al. [42] concluded that benzene, ethanol or methanol were the solvents of choice for the detection of ambient air particulate mutagenicity in the Ames test.

These studies (Tables I and II) suggest that acetone was the solvent-of-choice among those used for removal of organic compounds from sidestream cigarette particles. Use of methanol or dichloromethane as an extraction solvent greatly reduced the ability to detect mutagens with TA100.

Effect of refluxing

Goto et al. [42] cited the results of reflux experiments in their choice of extraction solvent. Increases or decreases in mutagenic activity were seen when several mutagenic pure compounds were refluxed in various extraction solvents. Several of the solvents evaluated by these investigators resulted in significant changes in the mutagenic activity of pure compounds when these compounds were refluxed in the extraction solvent, including dichloromethane and acetone. The results obtained in this study indicate that refluxing extracts of sidestream cigarette smoke particles does result in an apparent decrease in mutagenic activity in TA98 with metabolic

activation (Table III). Statistical analysis, however, does not demonstrate that this difference is significant. Activity in TA100 without metabolic activation, however, was significantly reduced after refluxing (Table III). These results suggest that the compounds responsible for the mutagenicity in TA100 may be compounds which are volatile or labile and, therefore, are lost during the refluxing process. Another possibility is that a chemical reaction occurs during the refluxing process which results in the transformation of the mutagenic compounds into compounds which are not mutagenic in the Ames test.

While differences are seen in the mutagenicity of replicate samples of sidestream cigarette smoke particles extracted with the 3 solvents compared in this study, these differences are generally not significant. The one significant difference is in the detection of direct-acting mutagens from sidestream cigarette smoke particles when acetone was used (Table II). Extraction using acetone resulted in higher levels of activity than seen with either dichloromethane or methanol.

Effect of storage

It has been reported that cigarette smoke condensate stored at refrigerator temperatures showed no loss of activity after 40 days; however, a 50% drop in activity in TA1538 was reported after the same length of storage time at room temperature [17]. The effect of storage of cigarette smoke particles or extracts on the mutagenicity of these substances was investigated in this study. Substantial reductions in the mutagenic activity of sidestream cigarette smoke particle extracts were observed after storage as either particles or as extracts in DMSO (Table IV). After storage for 60 days at −70°C in DMSO, the activity in TA98 with metabolic activation was reduced to 30% of the 'fresh' sample (less than 6 days storage). Greater reductions in activity were observed in TA100 without metabolic activation, as the samples contained only 12% of the activity of the 'fresh' samples. Storage as particles for 180 days at −70°C resulted in a 33% reduction of activity in TA98 with metabolic activation compared to the 'fresh' sample. These findings suggest that the effect of sample storage should be considered when attempting to evaluate the mutagenic activity of tobacco smoke particles. In general, it would seem prudent to conduct bioassay of the samples as quickly as possible after collection.

CONCLUSIONS

This study clearly demonstrates the mutagenicity of sidestream cigarette smoke particle extracts in the Ames *Salmonella* mutagenicity assay. The level of mutagenic activity of sidestream cigarette smoke particles was estimated from the slope of a dose-response curve calculated using the method of Stead et al. [11]. The number of revertants in the Ames test was estimated to be 15 000–20 000 per cigarette in TA98 with metabolic activation and 12 000–17 000 per cigarette in TA100 without metabolic activation. After refluxing acetone extracts of sidestream cigarette smoke

particles, the mutagenic activity in TA100 without metabolic activation was significantly reduced compared to extracts which were not refluxed.

Sidestream cigarette smoke particles were collected under experimental conditions by burning cigarettes in a chamber and collecting the smoke particles on glass fiber filters. The filters were extracted using either Soxhlet extraction or ultrasonic agitation and one of 3 solvents (dichloromethane, methanol, or acetone). When the combinations of extraction method and solvent were compared for their efficiency in detecting mutagenic activity in the Ames test, the differences in mutagenic activity were slight; however, ultrasonic agitation produced results which were more internally consistent than results with Soxhlet extraction. Of the 3 solvents which were compared, acetone yielded statistically higher levels of activity than the other 2 solvents (dichloromethane and methanol) in TA100 without exogenous activation. Differences in mutagenic activity in TA98 with metabolic activation among the 3 solvents, however, were small.

The effect of storing sidestream cigarette smoke particles and particle extracts on the mutagenic activity was also investigated. Activity in both TA98 with metabolic activation and TA100 without metabolic activation was found to be reduced after storage either as particles or as extracts in DMSO. Storage in DMSO at $-70°C$ for 60 days resulted in a reduction in mutagenic activity of 70% in TA98 with activation. Activity in TA100 without activation was reduced by 88% after the same storage. When stored as particles, the reduction in activity in TA98 was less (33%); however, the decrease in activity in TA100 was over 90%.

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